Biomedicine & Pharmacotherapy 90 (2017) 626–637

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www.em-consulte.com/en

Original article

Nigella sativa, a traditional Tunisian herbal medicine, attenuates bleomycin-induced pulmonary fibrosis in a rat model

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ARTICLE INFO

Article history:
Received 7 February 2017
Received in revised form 21 March 2017
Accepted 6 April 2017

Keywords:
Lung fibrosis
Nigella sativa oil
Metabonomics
Urine
TGFβ
Inflammation

ABSTRACT

The present study investigated the effects of *Nigella sativa* oil (NSO) on bleomycin (BLM)-induced lung fibrosis in rats. The rat model of pulmonary fibrosis (PF) was established by intratracheal instillation of BLM, and the effect of 1 ml/kg oral NSO treatment once daily observed. The effect of NSO was studied over a period of 50 days using ^{1}H RMN analysis on the urine and broncho alveolar lavage fluid (Balf) of the rats. Histopathological (inflammation and fibrosis) and immunohistochemical (TGF-β1 density) changes were evaluated.

Results found that the BLM group showed a significant increase in inflammatory index (II), fibrosis score (FS) and TGF-β1 distribution in the lung inflammatory infiltrate, accompanied by a decreased urinary secretion of Krebs cycle intermediates, including acetate, pyruvate, carnitine, trimethylamine-N-oxide and succinate. However, at the same time point, NSO treated rats had a reduced II and FS, and had an increased urinary secretion of histidine, fumarate, allantoin and malate.

In conclusion, NSO treatment attenuated the effects of BLM-induced PF, by supporting lung, liver and kidney activity in resisting PF. These findings provide an insight into the preventive and therapeutic potential of NSO in the treatment of PF.

1. Introduction

Pulmonary fibrosis (PF) is a chronic and incapacitating lung disease, which results in progressive scarring of the alveolar tissue that leads to trouble breathing [1]. In the characteristic pathological process of PF, normal tissue is replaced with mesenchymal cells and the extracellular matrix that these cells produce [2].

PF can be caused as a side effect of certain drugs such as bleomycin (BLM) or as a complication of autoimmune diseases, such as rheumatoid arthritis. BLM is an antineoplastic agent used for the treatment of a number of tumors. Frequent and repeated administration of BLM can cause lung inflammation, which can eventually progress into fibrosis [3]. The first sign of a fibrous lesion caused by BLM is a strong inflammatory response, which involves neutrophils, macrophages and T cells [4].

In response to inflammation and oxidative stress, a number of cytokines and inflammatory mediators are released by the damaged lung tissue, stimulating the formation of myofibroblasts and the accumulation of collagen in the extracellular matrix [5]. The primary treatment for PF is corticosteroids with a combination of immunosuppressant, anti-inflammatory, anti-fibrosis, antioxidant and anticoagulant drugs. However, treatment success is limited [6]. In a clinical trial, treatment with interferon-γ reduced the mortality rate of patients with PF, although it did not show any direct effect on fibrosis [7]. Consequently, finding an effective treatment for PF remains a challenge, and the development of novel antifibrotic drugs is vital [8]. Recently, experimental models of BLM-induced lung fibrosis have been used to investigate potential anti-fibrotic agents, including pirfenidone [9], PG490-88 [10] and LLDT-8 [11]. Despite the fact that these agents originate from different backgrounds (pirfenidone is a pyridine ketone, and PG-490-88 and LLDT-8 are derivatives of triptolide) they possess similar anti-fibrotic, antioxidant and anti-inflammatory activities. However, the exact mechanisms through which PG-490-88 and LLDT-8 provide protection against lung fibrosis remain unclear and

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http://dx.doi.org/10.1016/j.biopha.2017.04.009
0753-3322/

their application is limited by strong toxicity. Due to powerful antioxidant and anti-inflammatory activities, a number of natural agents, such as *Nigella sativa,* have long been used in traditional medicines to treat various diseases [12,13].

N. sativa, called 'habat al baraka' or 'al Habba al saouda' in the Middle East and 'Sinouj' in Tunisia, is a grassy plant belonging to the *Ranunculaceae* family with green and blue flowers and small black seeds. The black seeds have long been used in the Middle East and the Far East as a food preservative and spice. Furthermore, the seed and oil extracted from it have been used as a nutritional supplement. *N. sativa* oil (NSO) is used as a natural remedy for numerous diseases and symptoms, such as headaches, fevers, dizziness, inflammation, coughs, bronchitis, eczema, asthma, hypertension [14], diabetes [15] and gastrointestinal disorders [16]. Clinical and animal studies have identified that extracts of *N. sativa* seeds possess numerous therapeutic properties, including immunomodulatory, antibacterial, hepatoprotective, antihistaminic, antioxidative and neuroprotective effects [15].

NSO contains >100 components, including enzymes, vitamins, trace elements and aromatic oils [17]. Previous studies have reported that oral NSO treatment reduces the severity of bronchial asthma and allergic rhinitis [18,19]. In addition, previous pharmacological studies of NSO have shown that it has anti-inflammatory and immunomodulatory effects [20,21]. Recent studies have indicated that NSO has an inhibitory effect on systemic cytokines levels [22,23]. However, local immune responses have been reported to be more important than systemic immune responses in the induction of allergic inflammation in the lungs in a mouse model of allergic asthma [18,19,24]. Therefore, the present study investigated the anti-inflammatory and immunomodulatory effects of oral NSO on BLM-induced PF in male wistar rats, in regards to urinary and broncho alveolar lavage fluid (Balf) specific metabolites, and immunohistochemicl and histopathological changes of lung tissues.

2. Materials and methods

2.1. Extraction of N. sativa oil

N. sativa seeds were purchased from the traditional market of Blat Tunis (Tunis, Tunisia). To the best of our knowledge, the seeds were from Monsatir (Tunisia), where they were sown in the spring and harvested in late August. NSO was extracted using the method described by Folch et al. [25] and modified by Bligh and Dyer [26]. Briefly, the seeds were hand cleaned to remove the foreign materials, dried for 12 h at 105° C and crushed with a mixture of $HCCl_3$ and MeOH (2:1, v/v). The resulting powder was stored in a vacuum dryer until required. When needed, the crushed seed powder (10 g) was mixed with *n*-hexane (1:5, m/v) in a flask and put into the water bath at a controlled temperature at ~25° C. The solution was filtered through Whatman paper under a vacuum and concentrated with a rotary evaporator at 40° C to acquire NSO [27]. The NSO was further dried in a vacuum dryer to remove the residual *n*-hexane.

2.2. Experimental animals

Thirty-five male Wistar rats (weight, 350 ± 10 g; ~4 months old) provided by the animal farmhouse of the University of Mons (Mons, Belgium), were cared for according to the principles of the Ethics Committee on Animal Welfare of the University of Mons. Prior to group allocation and treatment, rats were acclimatized for 7 days in plastic cages. Then, rats were placed in metabolic cages in a well-ventilated room at a temperature of 22 ± 2° C, relative humidity of 50 ± 10% and light/dark cycle of 12 h. Rats had *ad libitum* access to food and water. At the end of the experiments rats were sacrificed by injection of sodium pentobarbital (100 mg/kg; Nembutal, Ceva Animal Health, Ltd., Amersham, UK).

2.3. Study design

Rats were randomly divided into seven groups of n = 5 (Table 1). The control group (G_1) was subjected to oral gavage with distilled water at a dose of 1 ml/kg/day over 50 days and sacrificed the next day. The second group (G_2) underwent a single BLM-induction (2 mg/kg in 200 μl saline) of fibrosis (described below) and was sacrificed three days later. The third group (G_2^1) underwent BLM-induction (2 mg/kg in 200 μl saline) of fibrosis and was sacrificed 20 days later. The fourth group (G_3) was subjected to oral gavage with NSO at a dose of 1 ml/kg/day for 30 days, was instilled with saline (2 ml/kg) intra-tracheally on the 31st day of treatment and sacrificed two day later. The fifth group (G_3^1) was subjected to oral gavage with NSO (1 ml/kg/day) for 30 days, was instilled intra-tracheally with saline (2 ml/kg) on the 31st day and sacrificed 20 days later. The sixth group (G_4) was subjected to oral gavage with NSO (1 ml/kg/day) for 30 days, instilled with BLM (2 mg/kg in 200 μl saline) intra-tracheally to induce fibrosis on the 31st day of treatment and sacrificed two days later. The seventh group (G_4^1) was subjected to oral gavage with NSO (1 ml/kg/day) for 30 days, instilled with BLM (2 mg/kg in 200 μl saline) intra-tracheally the next day, NSO treatment continued for a further 20 days more and then sacrificed the next day.

2.4. Bleomycin-induced PF

To investigate the presence of biomarkers associated with different stages of PF, groups were studied at a number of times prior to BLM induction of fibrosis (day −30) of fibrosis, once installed (day 1), at the beginning of the inflammatory phase (days 3 and 7) and during the fibrosis phase (days 14 and 21). In addition, a control group treated with saline (2 ml/kg/day) was followed for 21 days. On the day of instillation the rats were anesthetized by intraperitoneal injection of ketamine hydrochloride (87.5 mg/kg; Ketalar Pfizer, Inc., New York, NY, USA) and xylazine hydrochloride

Table 1
Study design.

Group	Treatment	Instillation (day 0)	Total Timing (days)	Sacrifice (days after instillation)
G_1	Distilled water	–	20	–
G_2	–	BLM	2	2
G_2^1	–	BLM	20	20
G_3	NSO	Saline	32	2
G_3^1	NSO	Saline	50	20
G_4	NSO	BLM	32	2
G_4^1	NSO	BLM	50	20

NSO, *Nigella sativa* oil; BLM, bleomycin.